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Neuropeptide Y in trigeminal ganglion following chronic constriction injury of the rat infraorbital nerve: is there correlation to somatosensory parameters?

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Abstract

The aim of this study was to investigate neuropeptide Y (NPY) levels in trigeminal ganglia following infraorbital nerve injury. Two experimental procedures were performed in three groups of rats: a unilateral chronic constriction injury (CCI) to the infraorbital nerve ($n = 13$), nerve manipulation without CCI ($n = 13$) and unoperated controls ($n = 8$). All rats underwent baseline and regular assessment of mechanical withdrawal threshold (Von Frey) and reaction to pin prick as well as free behavior evaluations. CCI to the infraorbital nerve induced significant hyperalgesia and allodynia within 9–12 days. At 6 days seven rats were euthanized and trigeminal ganglia harvested for immunocytochemical (ICC) studies. The study was ended at 14 days when all rats were euthanized and their ganglia harvested for ICC and radioimmunoassay (RIA) studies. An increase in NPY levels was seen in the ipsilateral ganglia of manipulated and CCI rats at 6 days, when rats displayed no pain-related behavior. At 14 days, ICC and RIA both detected significant increases in NPY levels in the ipsilateral ganglia of CCI and manipulated rats but not in unoperated controls. The possible roles of NPY in pain modulation and nerve injury are discussed in light of these findings.

Keywords: Neuropeptide Y; Trigeminal ganglia; Infraorbital nerve; Orofacial pain

1. Introduction

The three branches of the trigeminal nerve, the ophthalmic, maxillary and mandibular nerves, innervate the face of the rat. The maxillary division supplies the post orbital skin, the upper lip, mystacial vibrissae, lateral nose and the teeth and mucosa of the upper jaw (Waite and Tracey, 1995). The largest branch of the maxillary nerve is the infraorbital nerve, which is made up solely of sensory fibers. This branch supplies the mystacial pad and vibrissae that are richly innervated; each follicle receives some 250 nerve fibers of which one third are unmyelinated (Klein et al., 1988). The cell bodies of these fibers are located in the trigeminal ganglion (TG) that lies on the base of the skull, in the middle cranial fossa. At the ganglion, the ophthalmic and maxillary branches are indistinguishable in the rat.

The infraorbital nerve-injury model is based on results obtained from constriction of the sciatic nerve of the rat (chronic constriction injury, CCI) (Bennett and Xie, 1988), an experimental model of human neuropathic pain. Rats that have undergone sciatic nerve constriction develop allodynia (aversive behavior to a previously innocuous stimulus), and hyperalgesia (an exaggerated response to a normally noxious stimulus). Molecular parameters measured in the spinal cord following sciatic nerve CCI, such as gene expression, have correlated well with observed behavioral changes. However, sciatic nerve contains both sensory and motor fibers and constriction affects both groups of fibers. Furthermore it may not be a suitable model to study chronic pain in the face whose structures are innervated by the fifth cranial (trigeminal) nerve that may have different response properties to injury (Tal and Devor, 1992). Chronic constriction of the infraorbital nerve in the rat has been shown to cause behavioral (Vos et al., 1994; Imamura et al., 1996) and molecular changes (Vos and Strassman, 1995) indicative of persistent pain. Behavioral changes in this model have been previously assessed by the study of videotapes to measure alterations in locomotion and grooming behavior (Vos et al., 1994). In addition rat's responses to various mechanical stimuli were assessed,

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and later correlated with changes in c-fos expression in medullary dorsal horn (Vos and Strassman, 1995). Chronic constriction injury of the infraorbital nerve (IOCCI) results in a pain model applicable to the study of chronic neuropathic facial pain.

Neuropeptide Y (NPY), a 36 amino-acid peptide belonging to the pancreatic polypeptide family, is one of the most abundant peptides in the body and is conserved across various species (Wettstein et al., 1995). Since its discovery in 1982, NPY has been implicated in numerous homeostatic mechanisms mediated by a growing number of NPY receptors found in both peripheral and central nervous systems (Wan and Lau, 1995). The best known roles of NPY are in the control of systemic blood pressure, in memory, feeding, and anxiety. More recently a major role for NPY in mediating hyperalgesia and analgesia, with distinct peripheral and CNS mechanisms, has been demonstrated. NPY binding sites have been identified in neurons from dorsal root ganglia (DRG) and in TG (Mantyh et al., 1994). In naive animals virtually no NPY immunoreactivity (Ir) can be detected in dorsal root ganglion, but a significant increase in NPY is detected following nerve injury (Wakisaka et al., 1991). The intensity and staining pattern of DRG NPY Ir, as measured with immunocytochemistry, in a rat mononeuropathy has been positively correlated to measures of hyperalgesia (Munglani et al., 1995b). In the TG, NPY Ir is seen in the sympathetic perivascular fibers and is absent following cervical sympathectomy (Uddman et al., 1984). NPY Ir can however be detected in the TG following dental injury (Itotagawa et al., 1993), peripheral axotomy (Wakisaka et al., 1993), and following inferior alveolar nerve CCI (Wakisaka et al., 1996).

Our primary aim was to examine changes in neuropeptide Y content in TG following infraorbital nerve constriction at the expected onset of hyperalgesia. Additionally we examined NPY content in TG at the 6th postoperative day, when animals are generally not exhibiting pain behavior. By contrasting these results to those in control and in nerve manipulated rats we might shed further light on the possible role of NPY in nerve injury and facial pain modulation. Early findings have been presented in abstract form (Benoliel and Iadarola, 1999).

2. Materials and methods

2.1. Experimental animals

Male Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing 275–300 g (at time of surgery) were used. The NIDCR Animal Care and Use Committee approved all procedures and experimental protocols. Rats were habituated pre-operatively by allowing them 5–10 min in the sensory-testing apparatus for 4 consecutive days. During this time the rats were tested in the area of infraorbital nerve innervation with Von Frey hairs and a pin, then weighed and returned to their cages.

2.2. Treatments and surgical procedures

The study design included three groups, one active experimental group that underwent unilateral (left) nerve constriction ($n = 13$) and two control groups. In the first control group a unilateral surgical procedure was performed including release of the nerve from the surrounding connective tissue but no constriction (nerve manipulation, $n = 13$). Unoperated rats that would undergo exactly the same behavioral testing made up the second control group (control, $n = 8$). At postoperative day 6, seven rats were randomly chosen (three CCI, three manipulated, one control) and perfused for immunocytochemical (ICC) studies. These rats were not included in the behavioral analyses performed at the 14th postoperative day (pod). The end point of the study was set at 14 days when rats are usually hyperresponsive to infraorbital mechanical stimulation (Vos et al., 1994; Vos and Strassman, 1995). At this time rats were euthanized for ICC and radioimmunoassay (RIA) studies, as described below.

For surgical procedures rats were routinely anaesthetized with 0.3 ml of a mixture of equal volumes of ketamine (100 mg/ml) and xylazine (20 mg/ml) administered intraperitoneally. For transcardial perfusions the rats were euthanized with 500 mg/kg of pentobarbital sodium. Chronic constriction injury (CCI) was performed based on the original description by Bennett and Xie (1988) and the adaptation of Vos et al. (1994). In brief, rats were set up in a stereotaxic frame and a midline scalp incision was made to expose skull and nasal bones. The edge of the orbit formed by the maxillary, frontal, zygomatic, and lacrimal bones was dissected and subperiosteal access gained to the orbital floor. The rat's infraorbital nerve, which lies on the orbital floor, was accessible with mild retraction of the orbital contents and clearly visualized using a surgical microscope. At approximately 2–3 mm from the infraorbital nerve's most rostral portion, two loose chromic catgut ligatures (4/0), 1–2 mm apart were tied loosely. This caused minor constriction of the nerve such that the superficial vasculature is retarded but not totally occluded. The incision was closed with nylon sutures.

2.3. Behavioral testing

Tests were performed in the order they are described at the same time of day. The examiner was blinded as to the treatment group of each rat. To minimize effects of the examiner's presence we adopted recommendations made by Vos et al. (1994). The room was essentially dark and the area where the rat was tested illuminated by a 40-W light placed 1 m above. Rats were transported to the testing room in their cages, then individually transferred to a clear plastic cage with no bedding.

2.3.1. Exploration

Normal, naive rats explore their surroundings extensively by standing on their hindlimbs and performing numerous vibrissal movements. We used these behaviors as indicators of the total exploratory behavior that we expected to be affected following IOCCI. The total time spent performing 'vertical exploration' was recorded for a period of 7 min using a digital stop-clock. The total cumulative time recorded was approximated to the nearest second. We began timing vertical exploration when the rat's head went above a line drawn 12 cm from the floor of the cage and both forelimbs lost contact with the floor. The timer was stopped when the rat's head returned to below this line and at least one forepaw made contact with the floor. The height of the line was chosen based on pilot observations; when the rat stopped for grooming or other non-exploratory movements the head was below the line.

2.3.2. Grooming

To provide an indication of facial grooming we individually timed the duration and recorded the number of episodes the rat spent in performing any of the 15 facial grooming movements originally described by Berridge (1990), and adopted by Vos et al. (1994). The individual timings were approximated to the nearest second, these were then added to provide total time spent in facial grooming.

2.3.3. Allodynia

Mechanoallodynia was tested with Von-Frey monofilaments (Stoelting, IL). Prior to sensory testing, rats were habituated to the new situation for 5 min in a small clear plastic cage during which the operator reached into the cage very slowly with a Von Frey hair but did not contact the rat's face. The Von-Frey hairs were applied twice at intervals of 1–3 s, in order of increasing stiffness, to slightly different loci within the infraorbital territory. The first hair to evoke a clear withdrawal response was designated the threshold, for unmanipulated rats using our set of hairs this consistently occurred at 5.27 ± 0.36.

The monofilaments were not calibrated by us prior to use and we are therefore employing the markings as relative forces. The manufacturer provides the following equation to convert the von Frey markings to average forces in milligrams: Force in milligrams = (Antilog^{10} of marking)/10. Thus the 5.27 mean withdrawal response seen in unmanipulated rats is equivalent, on average, to a force of 18.62 g.

2.3.4. Hyperalgesia

Mechanohyperalgesia was assayed with a pinprick test adapted from published procedures (Tal and Bennett, 1994; Vos et al., 1994). The tip of a safety pin was pushed against the vibrissal pad, until the skin was dimpled but not penetrated. Scoring of the rat's response to this stimulus was on an ordinal scale: no response = 0, detection = 1, detection and withdrawal = 2, detection, withdrawal, and escape or attacking movements = 3, as in response 3 but with prolonged ipsilateral facial grooming (>3 strokes) = 4; a similar scoring system was used by Vos et al. (1994). Median response for unmanipulated animals was 2 (interquartile range = 0). The same test was applied to the skin below the ear (innervated by the auriculotemporal nerve). Following these tests the rats were weighed and returned to their home.

2.4. Immunocytochemistry

At 6 days seven rats were euthanized for ICC (three CCI, three manipulated, one control). At 14 days postoperatively nine rats (four IOCCI, four manipulated, one tested control) were randomly selected and euthanized. Two untested (naive) rats were also euthanized to provide control sections that would be run in parallel to the immunocytochemical sequences.

Transcardial perfusion was performed as previously described (Benoliel et al., 1999). The left and right trigeminal ganglia were harvested for ICC and the left and right infraorbital nerves excised and examined macroscopically. The ganglia were postfixed in the same fixative overnight, and transferred to 30% sucrose in PBS for 1–3 days for cryoprotection. Tissue sections (30 μm) were processed as floating sections using standard immunocytochemical protocols (Benoliel et al., 1999). NPY specific primary antiserum was used at a concentration of 1:3000 (Peninsula). Sections were mounted on gelatin-coated slides, dried overnight, cleared and coverslipped with Eukitt (Calibrated Instruments, Hawthorne, NY).

Distinct and intense immunoreactivity in the neuronal soma was counted in five sections from each ipsilateral rat ganglion in 14 rats euthanized for ICC. We limited the sections examined for cell counts to those from the central portion of the ganglia where the maximum biconvexity occurs and sections were grossly uniform in size. No sections from the edges of the ganglia or partial sections were included. Sections were from six rats that had undergone nerve manipulation, six rats that had undergone CCI (three sacrificed at pod 6 and three at pod 14 from each group) and two controls (one sacrificed at pod 6 and one at pod 14).

2.5. Radioimmunoassay (RIA)

Following CO_2 shock, the rats remaining (expected $n = 6$ control, six manipulated, six constricted, see results) were decapitated and both trigeminal ganglia rapidly harvested and frozen at −80°C. Both infraorbital nerves were dissected free and examined macroscopically. The ganglia were weighed, homogenized in 500 μl of 1 M acetic acid, two 100 μl aliquots were transferred to a clean test tube and lyophilized in a vacuum centrifuge. The pellets were reconstituted in 500 μl of RIA buffer by thorough vortexing, and following the manufacturer's protocol a RIA was performed to determine NPY content (Peninsula; Dwenger, 1984). In brief, the test tubes were incubated sequentially in NPY primary antibody, then radiolabelled NPY (^{125}I), both for 24 h each at 4°C. Samples were next incubated with goat anti-rabbit IgG and normal rabbit serum for 90 min at room temperature. Tubes were centrifuged at 3000 rpm and counts per minute (cpm) obtained from the pellet. The mean cpm for each duplicate was used to calculate the concentrations of NPY from the computer-generated standard curve and the content of NPY is expressed as femtomoles per ganglion or nanograms per gram of tissue for comparison with data in the literature.

2.6. Statistical methods

Alpha (two-tailed) for significance in all analyses was set at 0.05; when the Mann–Whitney U (MW) test was employed for pairwise comparisons alpha for significance was set at 0.01 to compensate for the multiple testing problem. Data was tabulated and analyzed using StatView 5 software (SAS Institute, NC).

2.6.1. Parametric

For vertical exploration and facial grooming timepoints of relevance were analyzed with a factorial ANOVA and a Student–Newman–Keuls (SNK) procedure (pairwise comparisons) to examine differences between individual groups. Where the ipsilateral and contralateral values were considered to be potentially different these were examined with a paired t-test. Percent change in body weight was calculated at days 6 and 9 (hyporesponsive) and at day 14 (hyperresponsive) relative to baseline and compared with a factorial ANOVA and a SNK.

2.6.2. Non-parametric

Grooming episodes, mechanohyperalgesia, mechanoallodynia and NPY content were examined with a Kruskal–Wallis test (KW) for significance between groups. This was followed by a MW for pairwise comparisons where indicated. If considered relevant, ipsilateral and contralateral data values within groups were compared with a Wilcoxon signed rank (WSR) test.

Cell counts obtained from the ICC sections were analyzed with a MW test.

3. Results

Data is expressed as the mean ± SD or as median with interquartile range (IQR). No large differences in baseline values between groups, for any of the parameters recorded, were noted. Analyses of behavioral data were performed on 26 rats (seven controls, 10 manipulated, nine constricted) for which we had complete data at all timepoints. One constricted rat that did not develop hyperresponsiveness and had macroscopical evidence of nerve transection was not included in any of the analyses. Behavioral changes relating to locomotor and grooming patterns were extensively studied and reported by Vos et al. (1994). We therefore limit ourselves to reporting and discussing these changes in brief, and use them mainly to corroborate changes in somatosensory parameters and in NPY concentration seen in the present study. These latter results are presented in detail in the figures and text.

3.1. Facial grooming

The distribution of grooming times was non-normal and the data was thus transformed for statistical analyses to a logarithmic scale that corrected this. Means were then transformed back for reporting.

At the 6th pod no differences were noted between groups in mean total time (s) spent performing facial grooming and in the median number of grooming episodes.

On the 14th postoperative day mean total time spent performing facial grooming was significantly higher in the nerve constriction group (63.39 ± 2.48) as compared to both the nerve manipulated (30.5 ± 1.76) and control groups (24.89 ± 1.58; factorial ANOVA: $F_{2,23} = 4.367$, $P = 0.0247$, SNK: $P < 0.05$). No significant differences were seen between the nerve-manipulated group and the control group at 14 days postoperatively (SNK: $P > 0.05$). No significant change from baseline grooming time values was noted in the manipulated or control rats. On the 14th postoperative day the median number of grooming episodes was significantly different between groups (KW test: DF = 2, corrected $H = 10.392$, tied $P = 0.0055$). Pairwise comparisons with a MW-U test revealed a significant difference between constricted (eight, IQR = 7.75) and manipulated groups (three, IQR = 3; $U' = 78.5$, tied $P = 0.006$), but none between manipulated and control groups (three, IQR = 1.75).

3.2. Vertical exploration

When hyperresponsiveness to sensory testing was clearly identified (14 days postoperatively) vertical exploration times (s) for the nerve constricted group (64.2 ± 29.09) were not significantly different than that observed in the manipulated (74.5 ± 26.44) or in the control groups (82.7 ± 30.05).

3.3. Mechanohyperalgesia

The immediate postoperative period was characterized by a lack of reaction to pinprick up to day 9 in the region innervated by the ipsilateral infraorbital nerve of constricted rats (Fig. 1). During this time manipulated rats reacted in a similar fashion to controls. This was comparable to that seen in measures of mechanoallodynia but shorter lasting, with hyperresponsiveness observed by the 12th postoperative day. By the 14th postoperative day constricted rats developed a significantly increased median response to pinprick on the ipsilateral side (three, IQR = 1.25) relative to manipulated (two, IQR = 0) or control rats (one, IQR = 1; KW: DF = 2, corrected $H = 15.532$, tied $P = 0.0004$). Pairwise comparisons of ipsilateral pinprick responses revealed that at 14 days the constricted group

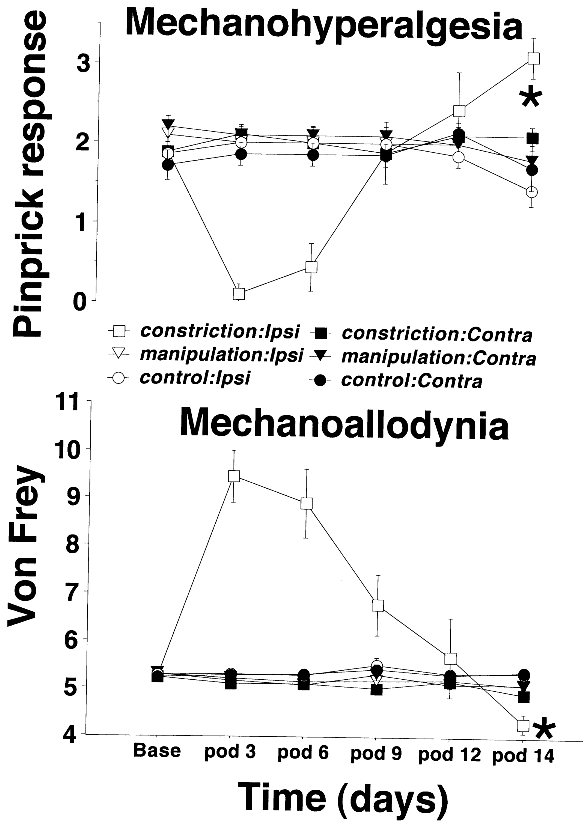

Fig. 1. Changes in response to pinprick following infraorbital nerve constriction CCI (top). The response to pinprick was subdued for 9 days following infraorbital CCI. By the 12th postoperative day (pod) most constricted rats had become hyperalgesic on the operated side (ipsi) and by day 14 this was statistically significant (*). No significant changes in response were observed in operated or unoperated (contra) sides from rats in the nerve manipulation or control groups. Changes in withdrawal threshold to Von Frey hairs following infraorbital nerve CCI (bottom). Postoperative hyporesponsiveness lasted for 12 postoperative days (pod) after which constricted rats developed significantly (*) reduced thresholds to mechanical stimulation by Von Frey hairs.

was significantly more hyperresponsive than the manipulated (MW-U test: $U' = 82$, tied $P = 0.001$). There was no statistical difference between the control and manipulated group ($U' = 48$, tied $P = 0.126$). For the contralateral side no significant differences were noted between any of the groups (KW: DF = 2, corrected $H = 2.955$, tied $P = 0.228$), at day 14. Comparison of ipsilateral and contralateral values within the constricted group revealed a significant difference (WSR test: tied $z = -2.201$, tied $P = 0.0244$). No differences between or within groups were noted for pinprick reactions in the area of the auriculotemporal nerve bilaterally (data not shown).

3.4. Mechanoallodynia

In the immediate postoperative period and lasting up to 12 days, rats in the nerve constriction group developed an absence of reaction to even the highest Von Frey hair available (6.65), and in these rats the response was graded as 10 to reflect this (Fig. 1). At 14 days, sensitivity had returned and a significant difference between groups was seen (KW: DF = 2, corrected $H = 13.09$, tied $P = 0.001$). At this timepoint the mean withdrawal threshold to Von Frey hair stimulation in the constricted group (4.29 ± 0.6) was significantly lower than in the control (5.34 ± 0.28. MW-U test: $U' = 60$, tied $P = 0.002$) and in the manipulated (5.07 ± 0.12) group (MW-U test: $U' = 76$, tied $P = 0.01$). No significant difference was observed between control and manipulated rats.

3.5. Weight

A significantly lower percent weight gain, relative to baseline, was noted in the nerve constricted group at 6-, 9- and 14-day periods (Factorial ANOVA: $P = <0.0001$, 0.0002, $P = 0.0006$, respectively, followed by a SNK). Only at day 6 was there a significant difference between manipulated and control, suggesting that the difference at this timepoint was also related to the degree of surgical trauma.

3.6. Macroscopic findings

Ipsilateral nerves excised from rats that underwent constriction showed edema on either side of the ligatures that caused a clear constriction injury. One rat, where the nerve was found to have been inadvertently transected, was excluded from all analyses.

3.7. Immunocytochemistry

Ipsilateral ganglia from rats obtained at pod 6 showed upregulation of NPY in manipulated and constricted rats but not in controls. NPY Ir was seen in large and medium cells but also in small neurons (Fig. 2). Cell counts revealed a mean of 146 ± 16 darkly stained cells in the constricted group and 88 ± 23 cells in the manipulated rats, but this did not attain statistical significance (MW: $U' = 8.0$, tied $P = 0.13$).

Ipsilateral ganglia obtained from constricted and manipulated rats at 14 days post-surgery showed a marked elevation of NPY Ir that was significantly greater in the constricted rats (MW: $U' = 9.0$, tied $P = 0.05$). Constricted rats still presented a large number of cells (cell count 158 ± 5) with intense NPY Ir, but this was not significantly different from the count obtained at pod 6 (MW: $U' = 6.0$, tied $P = 0.5$). The manipulated rats exhibited fewer cells that were intensely NPY Ir (cell count 50 ± 23, significantly lower than at pod 6, MW: $U' = 9.0$, tied $P = 0.05$), but there were many cells that were exhibiting medium to light Ir (Fig. 3). This pattern of NPY Ir was not seen in sections from tested controls, which were identical to those of naive untested rats. Contralateral ganglia in all animals contained detectable NPY Ir, with no differences between groups.

3.8. Radioimmunoassay

As previously stated, one rat from the constricted group was excluded due to macroscopic evidence of nerve transec-

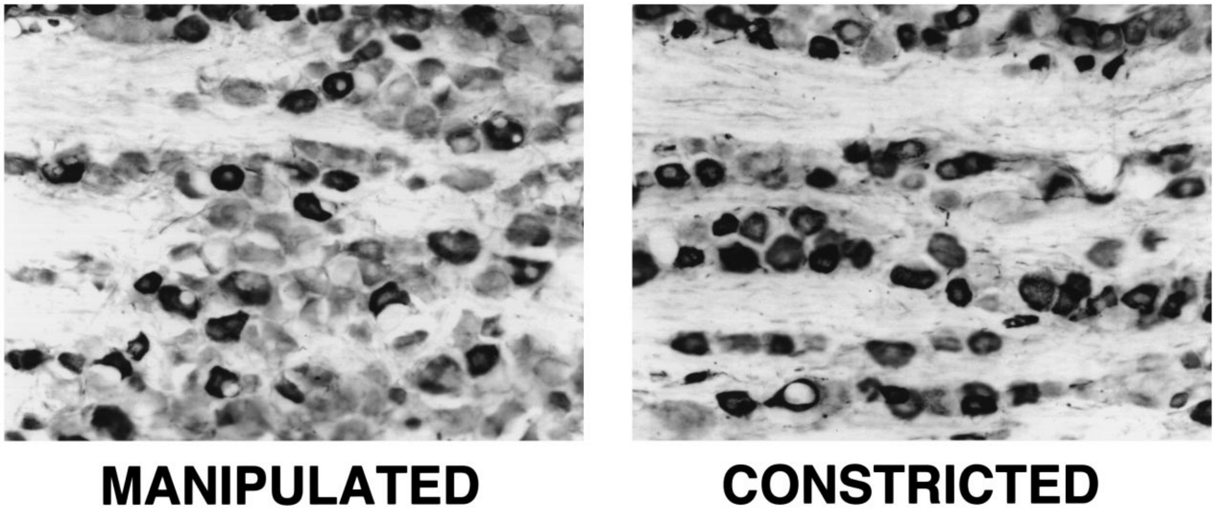

Fig. 2. NPY immunoreactivity (Ir) of trigeminal ganglia at 6 days postoperatively. A large number of cells demonstrate intense NPY Ir in both constricted and manipulated rats. Cell counts revealed a higher mean number of darkly stained cells in the constricted than in manipulated ganglia but this was not significant (see results). No difference in NPY Ir was seen between control and naive untested rats (not shown).

tion and persistent lack of response to mechanical stimulation (final $n = 17$). Mean weights of ganglia obtained from all rats were similar within groups (ipsi- versus contra-lateral), with no significant differences between treatment groups in the ipsilateral ganglia (factorial ANOVA: $F_{2,14} = 2.079$, $P = 0.162$). NPY content in each treatment group was not normally distributed and none of the commonly employed transformations was able to correct this. Additionally in view of the small sample size non-parametric statistical analyses were employed, however for clarity Fig. 4 shows the mean NPY content (±SEM). Median total NPY content of ipsilateral ganglia between groups was significantly different, (KW: DF = 2, corrected $H = 13.346$, tied $P = 0.0013$; see Fig. 4). MW-U pairwise comparisons of median NPY content of the ipsilateral side revealed that the constricted ganglia (164.57 fmol, IQR = 22.95) contained significantly more NPY than manipulated ganglia (95.34 fmol, IQR = 24.67; $U' = 30$, tied $P = 0.0062$). Manipulated ipsilateral ganglia contained significantly more NPY than control ganglia (8.64 fmol, IQR = 40.76; $U' = 34$, tied $P = 0.01$). No significant differences were observed for contralateral ganglionic NPY content between groups.

Scattergram plots of sensory parameters (pinprick response and von Frey thresholds) did not reveal a clear correlation pattern (Fig. 5).

4. Discussion

4.1. Experimental findings

An increase in NPY content in TG following constriction injury or physical manipulation of the infraorbital nerve was readily detected by ICC and in the RIA at both timepoints examined. In each case the increase was significant, but nerve manipulation did not elevate the peptide content as greatly as the constrictive nerve injury. At the 6 pod timepoint the NPY elevation in nerve manipulated and constricted rats occurred without changes in free behavior (e.g. feeding) or sensory parameters that would indicate a pain state. This would seem to indicate that in the early post-injury state NPY has a primary role in the response to nerve injury but not to pain. Although not attaining statistical significance, counts of cells with NPY Ir at pod 6 suggest that the degree of NPY upregulation is affected by the extent of nerve injury (CCI > manipulation). RIA and ICC data from the 14th pod further support this role for NPY. The manipulated rats had significantly elevated NPY in the ipsilateral ganglia despite the absence of pain behavior.

The TG of control rats revealed a measurable but small amount of NPY as assessed by ICC and RIA of the trigeminal ganglia. In a previous study NPY levels in DRG or TG of various species were found to be low (Roddy et al., 1990), but we found no data for the TG in rat. In the cat the mean baseline (NPY) in TG was reported to be 18 ± 6 (SEM) ng/g, comparable to median values of 3.76–6.63 ng/g found for contralateral ganglia in our study. These small amounts reflect the NPY content of autonomic sympathetic nerve fibers that are rich in NPY (Roddy et al., 1990; Uddman et al., 1984).

The present study shows the development of a decreased threshold to Von Frey stimulation, which we believe reflects mechanoallodynia, in parallel to the development of hyperresponsiveness to pinprick (mechanohyperalgesia). The sensory changes were nerve specific since we observed no change in response to pinprick from the auriculotemporal nerve. The study was ended at 14 days so we have no data to demonstrate what happens in the long-term to the allodynia. A reduced response to pinprick and an increased threshold to Von Frey hairs characterized the early postoperative period. This is probably due to the surgical trauma and is seen in other nerve injury models. The long delay to onset of hyperresponsiveness reflects the relatively more difficult surgical access to the infraorbital nerve.

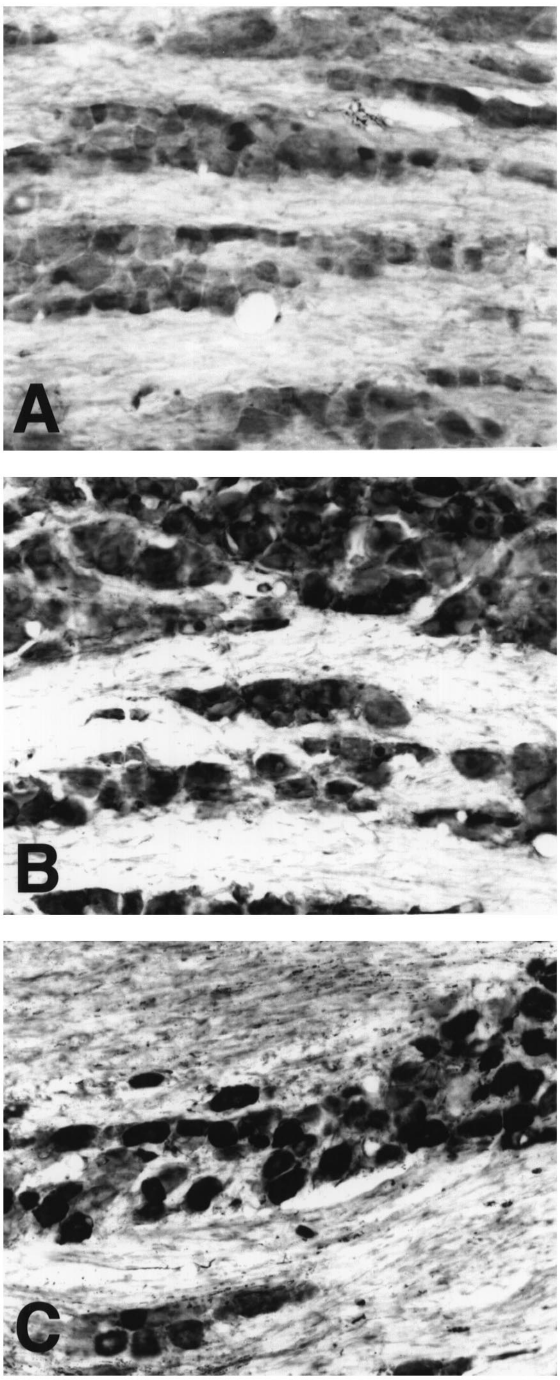

Fig. 3. NPY immunoreactivity (Ir) of trigeminal ganglia at 14 days post-operatively. Cells with NPY Ir are seen in manipulated (B) and constricted (C) rats. The intensity of NPY Ir was consistently higher in constricted rats than in manipulated rats. In the manipulated rats' sections the NPY Ir was diffuse with less cells showing distinct dark staining than that seen at 6 days. Cell counts revealed a significantly higher number of darkly stained cells in constricted relative to manipulated ganglia (see Section 3). In all sections NPY Ir was seen mainly in large and medium sized neuronal cell bodies. No difference in NPY Ir was seen between control (A) and naive untested rats (not shown).

We have also confirmed the behavioral changes following IOCCI previously observed by Vos et al. (1994). The use of facial grooming as an indicator of facial pain in experimental animals is complex. Vos et al. (1998) have shown that persistent directed facial grooming only follows noxious stimulation (formalin injection) but was absent following non-noxious sensory stimuli (local anesthetic nerve block, mineral oil, vibrissal clipping). However in animals following infraorbital axotomy (and thus analgesic) (Berridge and Fentress, 1986) and in our animals in the early postoperative period (analgesic to days 9–12), there is increased facial grooming in the absence of measurable pain. In long term observation facial grooming was maximal during the early postoperative period and waned as hyperresponsiveness increased (Vos et al., 1994), probably due to some effects of negative reinforcement (i.e. the grooming itself begins to cause increasing pain). We did not observe this due to the short nature of our experiment.

In the original description of the IOCCI model Vos et al. (1994) recorded an increase in 'freeze' behavior in constricted rats. Benoist et al. (1999) described expansion in the receptive fields of vibrissal afferents and changes in their cortical somatotopy but comment that these fail to result in major difficulties in thigmotaxic scanning employing current methodologies. In a similar fashion we noted non-significant differences in vertical exploration times between constricted and control groups and therefore conclude that this is not sensitive enough to be used in future trials. The constricted animals showed significantly reduced percent weight gain from baseline to 14 days suggesting disturbed feeding patterns, and intact trigeminal orosensa-

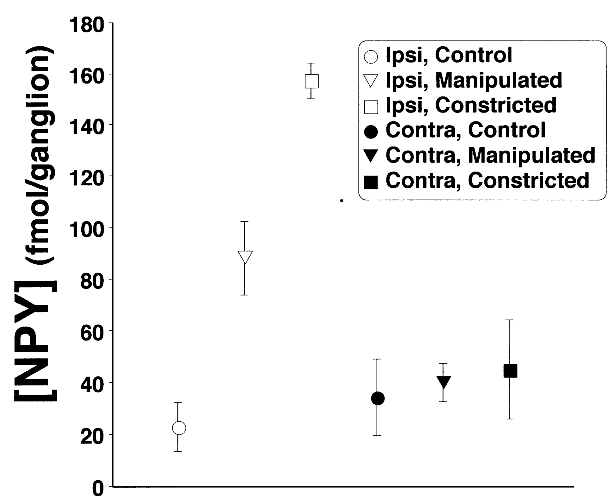

Fig. 4. Results of NPY radioimmunoassay at 14 days after infraorbital nerve CCI. Mean total amount (femtomoles) of NPY measured by RIA ([NPY]) was significantly increased in the ipsilateral (ipsi) ganglion of constricted and manipulated rats (*). No significant differences were observed in [NPY] found in contralateral (contra) ganglia between groups (see results). Although the data was analyzed nonparametrically we display mean ± SEM.

tion seems essential for normal ingestive behavior in the rat (Jacquin and Zeigler, 1983). Weight gain from baseline to the 9th postoperative also showed a significantly lower increase in the constricted group supporting the contention that it may be the nerve injury and disturbed sensation more than the ongoing pain that affects ingestive behavior.

4.2. Role of NPY in pain modulation

Actions of Y1 receptors are usually excitatory via actions on voltage-gated channels and release of intracellular calcium via inositol triphosphate. Y2 receptor activation inhibits extracellular calcium entry and is thus inhibitory. Under normal circumstances Y1 receptors are expressed in small DRG neurons and Y2 receptors in large DRG neurons. Following peripheral nerve injury Y1 receptor expression on small DRG cells decreases, but its expression is induced in some large neurons. The Y2 receptor is up-regulated both in small and large neurons (Zhang et al., 1994a). At the same time, there is a novel increase in NPY expression in medium to large DRG cells. The increase in NPY is solely in medium to large cells; neonatal capsaicin treatment that destroys C fibers does not affect post nerve injury increases in NPY mRNA expression (Noguchi et al., 1993). The appearance of NPY in these cells follows the time course of injury-induced Aβ firing (Frisen et al., 1992) that is thought to be related to the appearance of allodynia (Woolf and Doubell, 1994). However, at pod 6 we noted a number of small cells with intense NPY Ir. Walker et al. (1988) showed that NPY inhibits depolarization-induced release of substance P from DRG cells in vitro, and Duggan et al. (1991) have provided evidence for a similar action in vivo. In normal animals the Y1 receptor in DRG neurons is not transported into the spinal cord, and the effects of intrathecal NPY administration should thus either be mediated via Y1 receptors on local dorsal horn neurons, or on Y2 receptors on primary afferents from large CGRP-positive DRG neurons. The injection of NPY to the periaqueductal grey (PAG) resulted in prolonged withdrawal latencies to thermal and mechanical stimuli (Wang et al., 2000). This antinociceptive effect was reversed by administration of a Y1 receptor antagonist suggesting an important role for NPY in pain modulation in PAG, a structure that is important in nociceptive modulation.

There are also a limited number of Y2 receptor mRNA-positive local dorsal horn neurons. Given that large neurons are generally not associated with pain, any antinociceptive effect in normal animals should be related to the Y2 receptor containing interneurons (Y1 is usually excitatory). After axotomy Y2 receptors increase both in small and large neurons and intrathecal NPY may thus affect both these populations. In fact, in view of NPY's inhibition of Ca^{2+} influx and of substance P release (mostly co-stored with CGRP), and in view of the increased numbers of Y2 receptors on small neuron afferents, it is possible that the antinociceptive effect of NPY is enhanced following nerve injury. Together these results have been interpreted as an antinociceptive effect of NPY (Hokfelt et al., 1997).

In contrast, NPY has also been reported to inhibit Ca^{2+} influx into cultured rat DRG neurons via Y2 receptors (Ewald et al., 1988; Bleakman et al., 1991), and causes facilitatory and inhibitory effects on the nociceptive flexor reflex after axotomy (Xu et al., 1994). Additionally, NPY and the Y1 agonist (Leu31, pro34) NPY (Fuhlendorff et al., 1990) evoke an outward current in small DRG neurons at 1 μM (Zhang et al., 1994b, 1995), and in vitro studies on DRG cells have shown NPY to be excitatory (Abdulla and Smith, 1999). The excitatory effect of NPY was concentration-dependent and was amplified in cells taken from animals that had undergone axotomy (Abdulla and Smith, 1999). Taken together this may explain that in spite of the raised level of NPY in the manipulated rats this was of little sensory consequence due to: (1) relatively lower concentrations; and (2) the absence of injury-induced cellular changes in the DRG.

4.3. Peripheral actions of NPY and interactions with the sympathetic nervous system

The exact role of NPY in nerve injury-induced pain is unclear. Our findings would suggest a primary role for NPY in neuronal response to the injury itself; NPY was upregulated early after infraorbital nerve injury in both manipulated and constricted neurons in the absence of behavior that

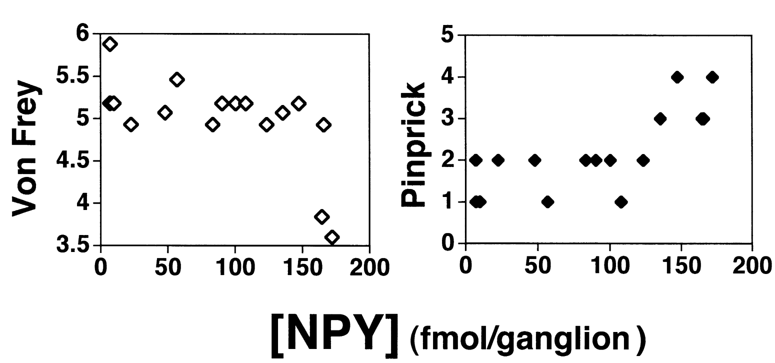

Fig. 5. Scattergrams of [NPY] values for all ipsilateral ganglia versus Von Frey thresholds (left) and pinprick responses (right) for the ipsilateral side in rats from the three groups ($n = 17$).

would indicate ongoing pain. Furthermore, based on our data we found no apparent correlation between NPY and mechanoallodynia at 14 days postoperatively (see Fig. 5). The correlation between NPY and hyperalgesia was unclear but the data suggest that there may be a level of NPY above which hyperalgesia appears (Fig. 5). The finding that hyperalgesia is somehow correlated to NPY levels has been previously reported in models of experimental spinal nerve injury but these studies analyzed NPY Ir at the onset of hyperalgesia only. At pod 14 there was still evidence of substantial NPY content in neurons from the manipulated rats. This is surprising in view of the mild character of the injury. However persistent NPY Ir in lamina 3–4 was seen 120 days following CCI of the sciatic nerve when hyperalgesia had resolved (Munglani et al., 1996), suggesting that neuropeptide plasticity persists in spite of resolving behavior.

There is some evidence however that peripheral release of NPY may play a critical role in neuropathic pain maintenance. Noradrenaline has been shown to increase the discharge rate of damaged neurons (Sato and Perl, 1991; Devor and Janig, 1981) as is seen in sympathetically maintained pain. The effects of noradrenaline are potentiated by NPY (Edvinson et al., 1984), an interaction enhanced in injured nerve (Frisen et al., 1992). The local administration of NPY to the paw of a rat with neuropathic pain resulted in increased mechanical and thermal hyperalgesia (Tracey et al., 1995), an effect abolished by sympathectomy. The different injury-related response properties of trigeminal afferents (Tal and Devor, 1992) and the lack of sympathetic invasion of TG following injury (Bongenhielm et al., 1999) questions the exact role that the sympathetic nervous system plays in the IOCCI model and needs investigating. Sprouting of sympathetic fibers (rich in NPY) into the DRG, such as is seen following other peripheral nerve injury (MacLachlan et al., 1993) was not found following injuries to the inferior alveolar and infraorbital nerves (Bongenhielm et al., 1999). This suggests that the increases in NPY seen following injury originate in TG cell bodies, which is confirmed by our ICC studies.

4.4. Is there a correlation between NPY levels and sensory changes?

A correlation between DRG NPY Ir and behavioral parameters of hyperalgesia has been demonstrated at 28 days post sciatic nerve CCI (Munglani et al., 1995a). The intensity of immunostaining was significantly reduced in rats pretreated with MK-801. Increased levels of NPY expression were also observed in lamina III of spinal cord dorsal horn, the area of myelinated sensory input related to the degree of peripheral hyperalgesia, at 28 days following nerve ligation (Munglani et al., 1995a). Again, pre-treatment with MK-801 suppressed both hyperalgesia and NPY expression in lamina III, but both these studies examine one timepoint only. In our study the results at the 6th pod timepoint indicate that in spite of no hyperalgesia NPY Ir is increased probably as a direct result of nerve injury. The RIA indicated no definite correlation between somatosensory parameters and NPY levels at pod 14. Moreover NPY was significantly elevated in the manipulated rats that had no significant pain behavior. Taken together our data suggest a primary role for NPY in the early neuronal response to injury. However from the scatterplots of pinprick responses versus NPY levels (Fig. 5) there is a suggestion that prolonged injury (as in CCI) may induce increased NPY levels that may then provoke a pain modulating role for NPY.

5. Conclusion

NPY is clearly upregulated (immunoytochemistry) in response to major nerve injury (CCI) and is also upregulated in rats subjected to minor nerve injury (manipulated) at a time (6th pod) when no sensory changes are seen in either group. After 14 days NPY was still significantly high (immunocytochemistry and radioimmunoassay) in nerve manipulated rats with no significant hyperalgesia suggesting a role for NPY in nerve injury per se. In the CCI rats NPY upregulation was highest and at these high concentrations NPY expression may be related to the degree of hyperalgesia. This study supports the in vitro data that NPY exerts its most potent effect in DRG cells following axonal injury and suggests interplay between altered peptide expression and primary afferent physiological activity due to nerve injury.

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